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RNT-1, the *C. elegans* homologue of mammalian RUNX transcription factors, regulates body size and male tail development

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Abstract

The *rnt-1* gene is the only *Caenorhabditis elegans* homologue of the mammalian *RUNX* genes. Several lines of molecular biological evidence have demonstrated that the RUNX proteins interact and cooperate with Smads, which are transforming growth factor-β (TGF-β) signal mediators. However, the involvement of RUNX in TGF-β signaling has not yet been supported by any genetic evidence. The Sma/Mab TGF-β signaling pathway in *C. elegans* is known to regulate body length and male tail development. The *rnt-1(ok351)* mutants show the characteristic phenotypes observed in mutants of the Sma/Mab pathway, namely, they have a small body size and ray defects. Moreover, RNT-1 can physically interact with SMA-4 which is one of the Smads in *C. elegans*, and double mutant animals containing both the *rnt-1(ok351)* mutation and a mutation in a known Sma/Mab pathway gene displayed synergism in the aberrant phenotypes. In addition, *lon-1(e185)* mutants was epistatic to *rnt-1(ok351)* mutants in terms of long phenotype, suggesting that *lon-1* is indeed downstream target of *rnt-1*. Our data reveal that RNT-1 functionally cooperates with the SMA-4 proteins to regulate body size and male tail development in *C. elegans*.

Keywords: Sma/Mab pathway; *dbl-1* pathway; Runt domain; PEBP2/CBF; TGF-β signaling

Introduction

Members of the transforming growth factor β (TGF-β) superfamily, which include the TGF-βs, activins, and bone morphogenetic proteins (BMPs), play important roles during development and growth. The dpp/BMP subfamily regulates many developmental events, including dorsal–ventral patterning, mesoderm induction, and osteogenesis (Irish and Gelbart, 1987; Panganiban et al., 1990; Wozney et al., 1988). For example, in *Drosophila*, *dpp*, *Gbb-60A,* and *screw*, which encode TGFβ-like ligands, are required during the embryonic and/or the pupal stages to regulate development (Arora et al., 1994; Chen et al., 1998; Khalsa et al., 1998; Padgett et al., 1987). Moreover, in *Caenorhabditis elegans*, the *dbl-1*, *unc-129*, and *daf-7* genes, which encode divergent TGFβ-like ligands, are involved in body length and male tail development (Krishna et al., 1999), axon guidance (Colavita et al., 1998), and dauer formation (Ren et al., 1996), respectively.

Two classes of receptor kinases for the TGF-β superfamily have been identified, namely, the type I and type II

Abbreviations: PEBP2, polyomavirus enhancer binding protein 2; TGF-β, transforming growth factor-β; *C. elegans*, *Caenorhabditis elegans*; GFP, green fluorescent protein; BMPs, bone morphogenetic proteins.

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receptor families. Upon ligand binding, the type II serine/threonine receptor kinase complexes with a corresponding type I receptor kinase and activates it by phosphorylation. The activated type I receptor kinase then transmits the TGF-β signal by phosphorylating receptor-regulated Smads (R-Smads), which are integral signal transducing elements in a variety of TGF-β pathways. Subsequently, the phosphorylated R-Smads complex with a common Smad (Co-Smad) and translocate into the nucleus. The R-Smad/Co-Smad complex then interacts with transcription factors, thereby regulating the expression of their target genes (Hanai et al., 1999; Liu et al., 1995; Miyazono et al., 1994; Ventura et al., 1994; Miyazawa et al., 2002; Weis-Garcia and Massague, 1996; Wrana et al., 1994).

In *C. elegans*, there are at least two well-characterized TGFβ-like signaling pathways. They are the Sma/Mab (small and male abnormal) and the Daf (dauer formation) pathways (Patterson and Padgett, 2000). The Sma/Mab pathway regulates body size and male tail development (Morita et al., 1999; Savage et al., 1996; Suzuki et al., 1999), while the Daf pathway regulates the formation of the dauer (alternative larval stage) when the worm population is excessive and/or food is scarce (Riddle and Albert, 1997). Interestingly, both pathways utilize the same type II receptor, DAF-4 (Krishna et al., 1999). As a result, the *daf-4* mutants have a small body size and a defective male tail, which are the typical consequences of mutations in Sma/Mab pathway components, as well as the constitutive dauer formation defects that result from mutations in Daf pathway elements.

The type I receptor (SMA-6, Krishna et al., 1999) and three Smad proteins (SMA-2, SMA-3, and SMA-4, Savage et al., 1996) that participate in *C. elegans* Sma/Mab pathway have also been identified. Mutations in these genes result in a smaller body size, fusion between rays 4 and 5, 6 and 7, and 8 and 9, and crumpled copulatory spicules in male tails (Krishna et al., 1999; Savage et al., 1996; Savage-Dunn et al., 2000). The downstream target genes, *lon-1* and *lon-3*, whose mutations result in a longer body than the wild type, were also identified in the Sma/Mab TGF-β signaling pathway (Maduzia et al., 2002; Suzuki et al., 2002). Recently, SMA-9 was reported as a transcriptional cofactor that confers specificity to Sma/Mab pathway (Liang et al., 2003). SMA-9 regulates the body size during early larval stages and formation of ray 9 in male tail, suggesting that other transcriptional factors need to regulate the body size at late postembryonic stage and other dopaminergic rays of male tail (Liang et al., 2003).

Polyomavirus enhancer-binding protein 2/core-binding factor (PEBP2/CBF) is a heterodimeric transcription factor that is composed of α and β subunits (Bae and Ito, 1999; Bae et al., 1994; Ogawa et al., 1993a,b; Wang et al., 1993). The α subunit contains a highly conserved 128-amino acid region that is termed the Runt domain (Kagoshima et al., 1993) after the *Drosophila runt* gene (Kania et al., 1990), and it binds directly to DNA. In contrast, the β subunit interacts with Runt domain and enhances the DNA binding of α subunit. There are three genes in mammals that encode the α subunits, namely, *RUNX1/AML1/PEBP2αB* (Bae et al., 1993; Miyoshi et al., 1991), *RUNX2/CBFA1/PEBP2αA* (Ogawa et al., 1993b), and *RUNX3/AML2/PEBP2αC* (Bae et al., 1995; Levanon et al., 1994). These RUNX proteins play important roles in both normal developmental processes and carcinogenesis. *RUNX1* is essential for definitive hematopoiesis (Okuda et al., 1996). This gene is located at the breakpoint of the chromosome translocations that are associated with human leukemia (Golub et al., 1995; Miyoshi et al., 1993; Romana et al., 1995) and is responsible for about 30% of human acute leukemia cases. (Look, 1997). *RUNX2* is required for osteoblast maturation and osteogenesis. Mice bearing a homozygous mutation in *RUNX2* died just after birth due to an inability to breathe that was caused by complete lack of ossification (Komori et al., 1997; Otto et al., 1997). *RUNX2* haploinsufficiency also causes the human bone disease cleidocranial dysplasia (Lee et al., 1997; Mundlos et al., 1997). *RUNX3* has been shown to be involved in neurogenesis of the dorsal root ganglia, T-cell differentiation (Levanon et al., 2002; Woolf et al., 2003), and tumorigenesis of gastric epithelium (Li et al., 2002; Guo et al., 2002). These RUNX transcription factors are also integral components of the signaling cascades that are mediated by both TGF-β and BMPs in several important biological systems. RUNXs bind to R-Smad and Co-Smad (Alliston et al., 2001; Hanai et al., 1999), and RUNX2 interacts with BMP-specific Smads and cooperates in inducing osteoblast differentiation (Zhang et al., 2000).

C. elegans has only one homologue of the mammalian *RUNX* genes, namely, *rnt-1* which has a very conserved Runt domain (Bae and Lee, 2000). This *rnt-1* gene has been reported to be expressed in the nuclei of hypodermal seam cells and intestinal cells (Nam et al., 2002). Interestingly, the RNA interference targeted to *rnt-1* gene shows the malfunction of the hypodermis and intestine, suggesting that *rnt-1* may be involved in the development of the hypodermis and intestine in *C. elegans*. Here, we reported another function of the *rnt-1* which is involved in regulation of body size and male tail development.

Materials and methods

C. elegans strains

The nematode *C. elegans* Bristol type (N2), CB1482 *sma-6(e1482)II*, LT186 *sma-6 (wk7)II*, CB502 *sma-2(e502)III*, CB491 *sma-3(e491)III*, PR675 *tax-6(p675)IV*, DR1369 *sma-4(e729)III*, CB185 *lon-1(e185)III*, PY1479 *kin-29(oy38)X*, and DR466 *him-5(e1490)V* were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota (St. Paul, MN). The KJ300 *cnb-1(jh103)V*

strain, which we isolated using a reverse genetics method (Park et al., 2001), has been reported previously by Bandyopadhyay et al. (2002). The VC200 *rnt-1(ok351)I* strain was isolated by the *C. elegans* Knockout Consortium. Worm breeding and handling were conducted as described (Brenner, 1974).

Body length measurement of adult worms

The *rnt-1(ok351)* mutant was outcrossed more than six times before any characterization was performed. Wild-type and mutant worms (20 each) were fed for 3–5 h at 20°C to induce the laying of embryos. When these F1 embryos became young adults after 90 h (72 h for the *rnt-1(ok351);lon-1(e185)* and *lon-1(e185)* mutants), animals were transferred to a 2% agarose pad and anesthetized with NaN_3 at a concentration of 50 mM for few minutes. Their body lengths were measured under a Nomarski microscope (Olympus BX50; Olympus, Tokyo, Japan).

The *rnt-1(ok351);lon-1(e185)* double mutants were easily ruptured when they became adult. It was difficult to measure the body length of adult *rnt-1(ok351);lon-1(e185)* mutants, because fully grown worms are more easily ruptured. To avoid this problem, we measured the body length of *lon-1(e185)* and *rnt-1(ok351);lon-1(e185)* at 72 h after being laid which was 18 h earlier than other double mutants to avoid the problem.

Characterization of male tail defects

To facilitate male tail analysis, each strain, including the *rnt-1(ok351)* mutant, was crossed with *him-5(e1490)V* to generate a double mutant (see below). The animals were propagated on NGM plates and harvested with M9 buffer, followed by three washes. The animals were then anesthetized with NaN_3 on 2% agarose pads and observed under a Nomarski microscope to score the defects in the spicules or rays.

Generation of double mutants bearing the rnt-1(ok351) mutation and a mutation in the Sma/Mab pathway gene

Double mutants containing mutations in the *sma-6*, *sma-2*, *sma-3*, *sma-4*, *tax-6*, *kin-29*, *cnb-1*, and *rnt-1* genes were constructed by crossing *rnt-1(ok351)* heterozygote males with the homozygous hermaphrodites of each mutant. The resulting F1 trans-heterozygous hermaphrodites were cloned, and heterozygosity for the *rnt-1* mutation was confirmed by PCR using primer sets described in Fig. 1. In the F2 generation, small mutants were carefully counted, and the phenotypic homozygotes were placed onto separate plates. Their *rnt-1* genotypes were confirmed by PCR. The Sma hermaphrodites that bore a *rnt-1(ok351)* homozygous gene were selected as double mutants.

Double mutants containing mutations in the *rnt-1*, *sma-6*, *sma-2*, *sma-3*, *sma-4*, and *him-5(e1490)* genes were constructed by crossing *him-5(e1490)* males with the hermaphrodites of each mutant. Non-Sma hermaphroditic progenies that resulted from these crosses were placed onto separate plates. The Sma hermaphrodites in the F2 generation were cloned on separate plates, and the plates bearing high frequencies of males were selected as double mutants.

Triple mutants bearing the *rnt-1(ok351), sma-[2*, *3*, *4*, or *6]*, and *him-5(e1490)* mutations were constructed by crossing *him-5(e1490)* males with the hermaphrodites of each double mutant [*rnt-1;sma-2*, *rnt-1;sma-3*, *rnt-1;sma-4*, or *rnt-1;sma-6*]. The non-Sma hermaphroditic progenies that resulted from the crosses were cloned onto separate plates. The Sma hermaphrodites that have a high frequency of males were selected as the *sma-[2*, *3*, *4*, *6];him-5* double homozygous animals. The *rnt-1* genotype of these animals was assessed by using specific primer sets, which allowed the triple mutant animals to be selected.

RT–PCR and sequencing

Total RNA was prepared from wild-type animals of mixed stages as described Sambrook and Russell (2001). The first strand of cDNA was synthesized by using 5 μg of total RNA and Sensiscript (Qiagene Cat. No. 205211) with the specific primer 5′-TAGTCGGAGAAGAACTATTC-GATGA-3′ according to the manufacturer's instructions. The single cDNA strand was amplified by PCR by using 5′-CTATCAATTTGGGCTGGAAATGACG-3′ as the upstream primer and 5′-AAAGTGCAGCTGAGATAGAAGG-3′ as the downstream primer. The amplified DNA fragment was subcloned into the pGEM-T easy vector (Promega, Madison, WI) and sequenced using the T7 and SP6 primers.

Transformation rescue and expression studies

To rescue the *rnt-1(ok351)* mutant phenotype, *rnt-1(ok351)* and *rnt-1(ok351);him-5(e1490)* mutant animals were coinjected with the *run-FL*::*gfp* fusion construct (Nam et al., 2002) along with the *rol-6* transformation marker at final concentrations of 50 and 100 μg/ml, respectively, as previously described (Mello and Fire, 1995). Individual Rol-6 and green fluorescent protein (GFP)-expressing progenies were then picked, and their phenotypes were examined.

Immunoprecipitation and immunoblot analysis

Two hundred ninety-three cells were transfected with *sma-4* tagged with the Myc epitope and *rnt-1* tagged with HA or Flag epitope, and lysed in ice-cold lysis buffer (25 mM HEPES [pH 7.5], 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 250 M phenylmethylsulfonyl fluoride, 10 g/ml leupeptin, 10 g/ml aprotinin) and cleared by centrifuga-

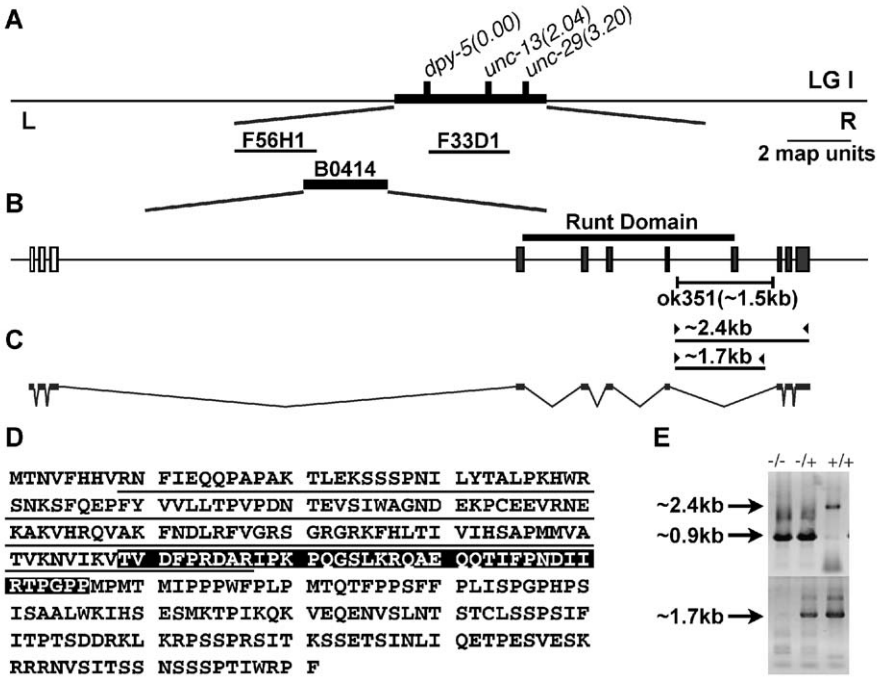

Fig. 1. Schematic representation of the *rnt-1* gene and the region that was deleted in the *rnt-(ok351)* mutant. (A) Genetic and physical maps of *rnt-1* in *C. elegans*. The *rnt-1* gene is located on chromosome I between the *dyp-5* and the *unc-13* loci of the genetic map and physically maps to the Cosmid B0414 (GeneBank accession number AF003145). (B) Gene structure of *rnt-1* and the region that was deleted in the *rnt-1(ok351)* mutant. The *rnt-1* gene is composed of 11 exons (indicated by boxes). The coding regions are represented by shadowed boxes. The upper line indicates the Runt domain, which is highly conserved among humans, mice, chicks, *Drosophila melanogaster*, and *C. elegans*. The lower lines indicate the region of the *rnt-1* genomic DNA that is deleted in *rnt-1(ok351)* mutant and the predicted lengths of the PCR products generated by each set of primers as indicated by the arrowheads. (C) The RNA transcripts of the *rnt-1(ok351)* mutant. The RT–PCR and sequencing experiment detected *rnt-1(ok351)* mRNA, which lacks the eighth exon of wild-type mRNA. (D) Amino acid sequence of RNT-1. The Runt domain is underlined, and the black box indicates the region of the protein that is deleted in the *rnt-1(ok351)* mutant. (E) Single worm PCR results. PCR bands obtained from single worms using the primer sets described in B were used to determine whether the animals were homozygotes (−/−), heterozygotes (+/−), or wild type (+/+). The absence of a 1.7-kb band in the lower panel (−/−) confirms that the animal is a homozygote.

tion. Immunoprecipitations were carried out by adding the appropriate antibodies plus protein G-sepharose beads, followed by incubation at 4°C. The immunoprecipitates were washed extensively, resolved by SDS–PAGE, and analyzed by Western blotting using the appropriated antibodies. Antibodies used for immunoprecipitation were anti-Myc (9E10, Santa Cruz) and anti-Flag (M2, Sigma).

Glutathione S-transferase (GST) pull-down assay

GST pull-down experiments were performed to test physical interaction between RNT-1 and SMA-4. A GST-fusion protein containing the full-length RNT-1 protein was expressed in bacteria and purified. Two hundred ninety-three cells were transfected with full-length *sma-4* tagged with the Myc epitope, and the cells were lysed in ice cold lysis buffer (25 mM HEPES [pH 7.5], 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 250 M phenylmethylsulfonyl fluoride, 10 g/ml leupeptin, 10 g/ml aprotinin). One milligram of the lysate was incubated with the glutathione–sepharose beads-bound GST–RNT-1 in 1 ml of ice cold lysis buffer containing 0.5% of the NP-40. The mixture was then washed vigorously five times with 1 ml of the same buffer. After boiling in the SDS sample buffer, the mixtures were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blotting using an anti-Myc antibody (9E10, Santa Cruz).

Results

The rnt-1(ok351) mutation bears a deletion

The *rnt-1* gene was previously found to encode the only *C. elegans* homolog of the mammalian RUNX proteins, which are the α subunits of the CBF/PEBP2 transcription factor (Bae and Lee, 2000). The *rnt-1* gene physically maps to Cosmid B0414 between the *dpy-5* and the *unc-13* loci on the cluster region of linkage group I (Fig. 1A). This gene was previously characterized and was found to contain 11 exons and to encode a 301-amino acid predicted protein (Figs. 1B, D) that contains the conserved Runt domain (Bae and Lee, 2000).

A *rnt-1* deletion mutant, *rnt-1(ok351)*, was isolated by the *C. elegans* Knockout Consortium (kindly provided by R. Barstead, Oklahoma), and this led us to further investigate the function of *rnt-1* in *C. elegans*. First, we

analyzed the region of *rnt-1* that was deleted in the *rnt-1(ok351)* mutant. The deletion removed approximately 1.5 kb of the *rnt-1* gene that spanned the eighth intron to the ninth intron (Figs. 1B, E). RT–PCR and DNA sequencing revealed that transcription generated *rnt-1* mRNA which was spliced exactly from the seventh exon to the ninth exon (Fig. 1C), which would result in the deletion of 38 amino acids, including 9 amino acids of the Runt domain (Fig. 1D). According to the recently solved X-ray crystal structure of the Runt domain, this deleted region of Runt domain corresponds to the C-terminal DNA-binding loop (Tahirov et al., 2001; Zhang et al., 2003). Therefore, this deletion mutation may affect the DNA-binding activity of RNT-1. However, given that the *rnt-1(ok351)* mutant still produces *rnt-1* mRNA, albeit in a spliced form, it is possible that the mutated RNT-1 protein may still retain functions that are independent of its DNA-binding activity (see Discussion).

For the further analysis of *rnt-1(ok351)* mutant as described below, homozygote animals were first identified by nested PCR. Homozygosity was indicated by a 0.9-kb DNA fragment produced by the primers located outside of the deletion region and the absence of a 1.7-kb band when the inner primers were used (Fig. 1E). The homozygous animals were then outcrossed more than six times to eliminate any other possible mutations.

The rnt-1(ok351) mutant has a small body size and male tail defects

Interestingly, the *rnt-1(ok351)* mutant did not show gross defects as adults or any developmental defects. However, when we observed the mutant more closely, we found that its brood size was slightly decreased compared to the wild-type animal, as the number of progeny per single worm was 274 ± 50 for the wild-type and 215 ± 63 for *rnt-1(ok351)*. We then measured the body length and found that the average body length of *rnt-1(ok351)* is 1.16 ± 0.10 mm, whereas that of the wild-type animal (N2) is 1.46 ± 0.07 mm (Table 1). This indicates that the body length of the *rnt-1(ok351)* mutant is approximately 20% smaller than that of the wild-type animal (Table 1 and Fig. 2).

To characterize the *rnt-1(ok351)* male in more detail, we generated a male population by using the *him-5(e1490)V* background (see Materials and methods). The male tail of wild-type worm has nine pairs of rays, which are chemosensory organs, and a pair of spicules, which is a bilaterally symmetric copulatory structure. Each of the nine pairs of rays, which are surrounded by round fan, is unique with respect to its morphology, position, and chemosensitivity. Interestingly, the tail of the *rnt-1(ok351)* male is severely deformed (Fig. 3), as the rays were frequently absent, fused, misaligned, or shortened compared to those of the control *him-5(e1490)* males. Furthermore, the shape of the fan surrounding the nine pairs of rays was quite irregular.

Table 1
The *rnt-1(ok351)* mutant and Sma/Mab mutants show synergistic effect for body length

Genotype	Body length	%	*n*	*P* value[a]
N2	1.46 ± 0.07	100	17	
rnt-1(ok351)	1.16 ± 0.10	79.4	48	<0.001
rnt-1(ok351);Ex[pPD-run-FL]	1.34 ± 0.08	92.1	46	<0.001
sma-6(e1482)	0.99 ± 0.11	66.1	48	
rnt-1(ok351);sma-6(e1482)	0.75 ± 0.11	50.2	22	<0.001
sma-6(wk7)	1.01 ± 0.08	68.0	44	
rnt-1(ok351);sma-6(wk7)	0.86 ± 0.08	57.9	45	<0.001
sma-2(e502)	0.84 ± 0.08	56.1	45	
rnt-1(ok351);sma-2(e502)	0.74 ± 0.09	49.9	13	<0.05
sma-3(e491)	0.90 ± 0.07	62.0	49	
rnt-1(ok351); sma-3(e491)	0.84 ± 0.12	56.6	45	<0.05
sma-4(e729)	1.01 ± 0.09	67.5	46	
rnt-1(ok351); sma-4(e729)	0.91 ± 0.06	61.0	49	<0.001
lon-1(e185)[b]	1.52 ± 0.15	124.6	32	
rnt-1(ok351);lon-1(e185)[b]	1.51 ± 0.25	122.8	18	>0.05
tax-6(p675)	0.96 ± 0.11	64.4	87	
rnt-1(ok351); tax-6(p675)	0.97 ± 0.08	66.6	48	>0.05
kin-29(oy38)	0.96 ± 0.08	64.4	44	
rnt-1(ok351); kin-29(oy38)	0.98 ± 0.07	67.3	47	>0.05
cnb-1(jh103)	0.94 ± 0.06	64.6	47	
rnt-1(ok351);cnb-1(jh103)	0.95 ± 0.06	65.2	50	>0.05

[a] The *P* values were calculated using Microsoft office Excel to show the statistical probability when compared to the numbers in the upper row.
[b] The body lengths were measured at 72 h after being laid, and the percentages of body length were calculated relative to wild-type body length at the same time.

To confirm that the small body size and the defective male tail are caused by the mutation in the *rnt-1* gene, *rnt-1(ok351)* mutant animals were injected with the pPD-run-FL construct, which contains the full-length genomic *rnt-1* DNA fused to the *gfp* (green fluorescent protein) reporter gene (Nam et al., 2002). Both the small body size and defective male tail phenotypes of the *rnt-1(ok351)* mutant animal were rescued by the pPD-run-FL transformation (Tables 1 and 2, and Figs. 2C and 3F). This indicates that these defects are specifically caused by the *rnt-1(ok351)* mutation.

The small body size and male tail defects of the *rnt-1(ok351)* mutant are very similar to the phenotypes of animals that bear a mutation in a Sma/Mab TGF-β signaling pathway component. An example of such mutants is the *sma-6(wk7)* mutant, which bears a mutation in the *sma-6* gene, the receptor kinase type I of the Sma/Mab pathway in *C. elegans* (Tables 1 and 2 and Figs. 2D and 3E). This resemblance encouraged us to investigate the relationship between RNT-1 and the Sma/Mab pathway.

rnt-1 is expressed in the male tail

rnt-1 was previously reported to be expressed in the nuclei of hypodermal seam cells and intestinal cells (Nam et al., 2002). However, the defects in the male tail of *rnt-1(ok351)* led us to monitor the spatial *rnt-1* expression pattern in the male tail by injecting the pPD-run-FL construct into *him-5(e1490)* animals. The GFP

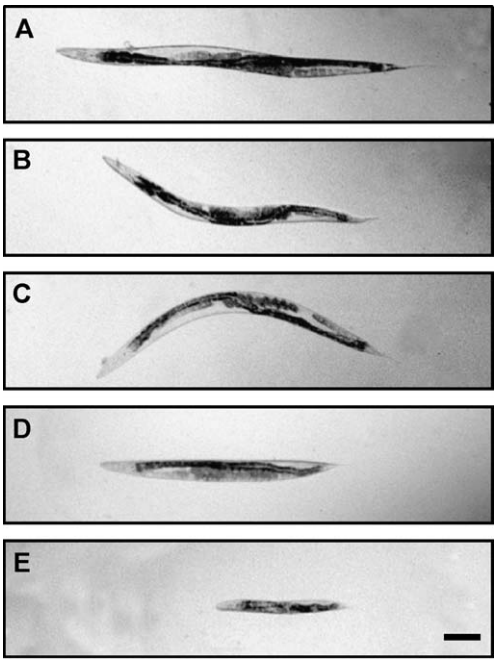

Fig. 2. Body size of the *rnt-1(ok351)* single and double mutants. (A) Nomarski image of wild type (100%), (B) *rnt-1(ok351)* (79.4% of the length of wild type), (C) *rnt-1(ok351)[pPD-run-FL]* (92.1%), (D) *sma-6(wk7)* (68%), and (E) *rnt-1(ok351);sma-6(wk7)* (57.9%) animals. The animals are all shown at the same magnitude. Bar indicates 20 μm.

reporter protein was indeed detected in the descendant cells of the V5, V6, and T lineages, which include all the A-type neuron, the B-type neuron, and the structural cells of the rays from the mid-L3 stage to L4 stage (Figs. 3G and H).

The Sma/Mab TGF-β signaling pathway genes in *C. elegans* have been reported to be expressed in the hypodermis, intestine, pharynx, and male tail. This has been observed, for example, for the *sma-6* gene that encodes the type I receptor specific for the Sma/Mab pathway (Krishna et al., 1999) and for the *sma-3* gene that encodes the cytosolic Smad signal transducer (Wang et al., 2002). These expression patterns overlap well with the *rnt-1* expression pattern apart from the pharyngeal expression. Taken together, our observation suggests that *rnt-1* may participate in the Sma/Mab pathway, which regulates body length and male tail development (Krishna et al., 1999; Savage et al., 1996; Savage-Dunn et al., 2000).

The rnt-1(ok351) mutant shows synergistic effects with known mutants of Sma/Mab pathway genes

To investigate the relationship between *rnt-1* and the genes that are known to be involved in the Sma/Mab pathway, genetic analyses were performed using several double mutants. Mutations in these known Sma/Mab pathway genes all result in small body size (Table 1 and Fig. 2D). Two mutant *sma-6* alleles have been identified, *e1482* and *wk7*, and these appear to be a loss-of-function mutant and a null mutant, respectively. Both alleles result in small body sizes (0.99 ± 0.11 mm for *e1482* and 1.01 ±

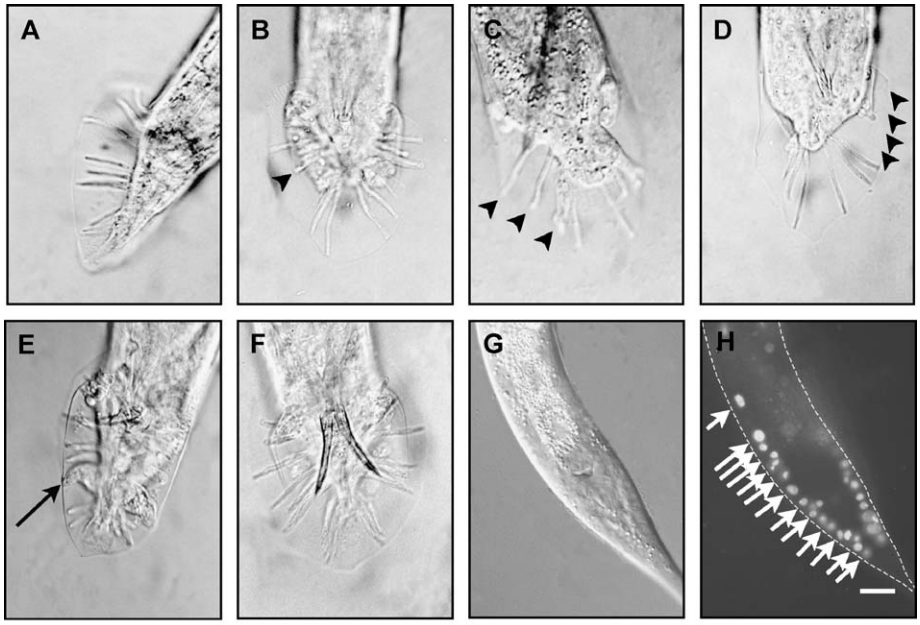

Fig. 3. Male tail defects of *rnt-1(ok351)* animals. (A) Nomarski image of *him-5(e1490)* shows nine pairs of normal rays. (B–D) Nomarski images of *rnt-1(ok351)* male tails show a shortened ray (indicated by arrowhead in B), fused rays, misaligned rays (indicated by an arrowhead in C), and missing rays (indicated by an arrowhead in D). (E) The *sma-6(wk7)* male tail shows fusion of rays 6 and 7, as indicated by a black arrow. (F) The *rnt-1(ok351)* mutant that has been rescued by transformation with the pPD-run-FL construct shows nine normal pairs of rays and a pair of normal elongated spicules. (G–H) GFP reporter gene expression pattern of *rnt-1*. G shows the Nomarski image of H. GFP was detected in the male tail (indicated by white arrows in H) in addition to the intestinal and hypodermal seam cells.

Table 2
The *rnt-1(ok351)* mutant and Sma/Mab mutant show the synergistic effect in male tail developments

Genotype	Frequency of ray fusion or ray missing (%)									% Abnormal spicule	*n* (Half sides)
	1	2	3	4	5	6	7	8	9		
him-5(e1490)	0	0	0	0	0	0	0	0	0	1	73
rnt-1(ok351);him-5(e1490)	2	2	15	70	70	47	8	0	0	25	53
rnt-1(ok351);him-5(e1490), Ex[pPD-run-FL:]	9	12	12	11	6	8	11	5	5	8	66
sma-6(wk7);him-5(e1490)	0	8	11	25	37	63	52	8	1	92	73
rnt-1(ok351);sma-6(wk7);him-5(e1490)	69	77	63	62	71	58	44	49	53	81	78
sma-6(e1482);him-5(e1490)	0	0	0	0	0	4	4	0	0	0	50
rnt-1(ok351);sma-6(e1482);him-5(e1490)	12	22	15	68	73	61	22	10	7	26	74
sma-2(e502);him-5(e1490)	0	0	1	14	16	85	85	14	11	91	74
rnt-1(ok351);sma-2(e502);him-5(e1490)	60	71	56	78	78	55	53	47	30	89	73
sma-3(e491);him-5(e1490)	2	2	2	12	32	74	60	11	4	95	54
rnt-1(ok351);sma-3(e491);him-5(e1490)	57	76	55	76	81	59	60	45	38	79	58
sma-4(e729);him-5(e1490)	1	1	0	10	18	83	81	13	5	91	77
rnt-1(ok351);sma-4(e729);him-5(e1490)	58	73	50	59	70	66	44	40	36	97	64

0.08 mm for *wk7*) (Krishna et al., 1999; Table 1). Surprisingly, the *rnt-1(ok351)I;sma-6(e1482)II* and *rnt-1(ok351)I;sma-6(wk7)II* double mutants have much smaller body sizes than either of the *rnt-1(ok351)*, *sma-6(e1482)*, or *sma-6(wk7)* single mutants (Table 1). The double mutants containing the *rnt-1(ok351)* mutation plus a mutation in the *sma-2*, *sma-3*, or *sma-4* gene also have much smaller body size than either the *rnt-1(ok351)* or *sma-2*, *sma-3*, or *sma-4* single mutants (Table 1). In contrast, double mutants containing the *rnt-1(ok351)* mutation plus a mutation in one of three genes known to be not involved in TGF-β signaling, namely, *tax-6(p675)IV*, *cnb-1(jh103)V*, and *kin-29(oy38)X*, do not have an enhanced decrease in body length (Table 1). The *tax-6* and *cnb-1* genes encode calcineurin A and B and are involved in G-protein-coupled signaling pathways (Bandyopadhyay et al., 2002). The *kin-29* gene encodes a serine/threonine kinase and is known to regulate chemoreceptor expression, body size, and dauer formation (Lanjuin and Sengupta, 2002). Mutations in these genes all cause a small body phenotype without male tail defects.

To confirm the effects of RNT-1 in the Sma/Mab TGF-β signaling pathway, we also observed the male tails of the double mutants bearing the *rnt-1(ok351)* mutation together with a mutation in *sma-2*, *-3*, *-4*, or *-6*. The *sma-6(wk7)*, *sma-2(e502)*, *sma-3(e491)*, and *sma-4(e729)* single mutants frequently show fusion between rays 4 and 5, 6 and 7, and 8 and 9, and crumpled copulatory spicules. The exception to this is the loss-of-function allele *sma-6(e1482)*, which does not show these male tail defects. As described above and shown in Figs. 3B–D, the *rnt-1(ok351)* mutant animals exhibit various male tail abnormalities, including the frequent absence of several rays, fused rays, and shortened rays. The shape of fans surrounding the rays is also irregular. However, the spicules are fully elongated and appear to be normal. When the *rnt-1(ok351)* mutation occurs together with a mutation in *sma-2*, *-3*, *-4*, or *-6*, the male tail defects are enhanced since more rays were deformed in the double mutants than in the single mutants, and the spicules were crumpled much more frequently (Table 2).

The phenotype of lon-1(e185), which is the downstream target mutant of Sma/Mab pathway, is epistatic to the rnt-1(ok351) mutant phenotype

The *lon-1* gene, which encodes a PR-related protein, has been previously identified as one of the downstream genes that regulate body length (Morita et al., 2002). Intriguingly, the expression of *lon-1* has been shown to be suppressed by the Sma/Mab TGF-β signaling pathway (Maduzia et al., 2002). LON-1 appears to be a negative regulator of body-length in *C. elegans* since a loss-of-function mutant, *lon-1(e185),* has a much longer body length (Table 1), while overexpression of *lon-1* has been shown to cause a small body size (Morita et al., 2002). To examine whether there is a genetic interaction between *rnt-1(ok351)* and *lon-1(e185)*, the *rnt-1(ok351);lon-1(e185)* double mutants were generated. At first, this *rnt-1(ok351);lon-1* double mutants appeared thin and long like "small snake," which looked very similar to *lon-1(e185)*. However, the body length of *rnt-1(ok351);lon-1(e185)* double mutants were little shorter than *lon-1(e185)* mutants when we measured body length at 90 h after being laid [1.61 ± 0.11 mm for *lon-1(e185)* and 1.47 ± 0.17 mm for *rnt-1(ok351);lon-1(e185)*]. Upon more careful examination, they had an additional phenotype. The *rnt-1(ok351);lon-1(e185)* double mutants were easily ruptured even in the NGM when they reached adulthood. This might have biased to measure somewhat smaller worms at 90 h after being laid, since many fully grown double mutants were ruptured dead. Thus, we remeasured the body length as they grow during developmental stages (Fig. 4). Interestingly, the *rnt-1(ok351);lon-1(e185)* double mutants showed the long phenotype which was identical to the phenotype of *lon-1(e185)* during development until 72 h after being laid, suggesting *lon-1(e185)* is epistatic to *rnt-1(ok351)*.

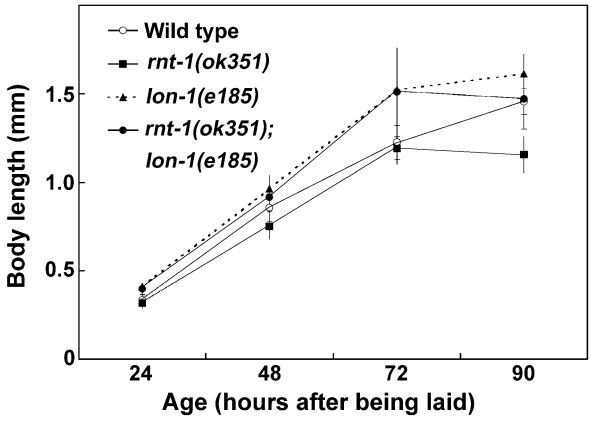

Fig. 4. Growth curves of N2 wild type, *rnt-1(ok351)*, *lon-1(e185)*, and *rnt-1(ok351);lon-1(e185).* The body lengths of mutants were measured at various time points after being laid. The measurement of the *rnt-1(ok351);lon-1(e185)* double mutants at 90 h after being laid was for nonruptured animals among viable worms.

RNT-1 physically interacts with SMA-4

To investigate any physical interaction between the Sma/Mab pathway components and RNT-1, SMA-4 and RNT-1 were transiently transfected into 293 cells and coprecipitated in two independent reactions (Figs. 5A, B). Changes of tagging and the antibodies for immunoprecipitation and Western blotting showed essentially the same result (Fig. 5B) which indicate that the RNT-1 physically interacts with SMA-4.

A GST pull-down assay was also performed. SMA-4 was expressed in a mammalian cell line, and the cell lysate was added to glutathione–sepharose-bound GST–RNT-1. An association between SMA-4 and GST–RNT was detected, while this association was undetectable when GST was used (Fig. 5C). Thus, RNT-1 can physically interact with SMA-4 in vitro.

Discussion

rnt-1(ok351) might be a reduction-of-function mutant

All three mammalian *rnt-1* homologues, the *RUNX* genes, are essential for survival and play important roles in hematopoiesis, osteogenesis, and suppression of gastric cancer. For instance, *runx1*$^{(-/-)}$ mice die around E12.5 due to the absence of the myeloid and erythroid progenitors of definitive hematopoietic origin in either the yolk sac or fetal livers (Okuda et al., 1996). This suggests that *RUNX1* is essential for definitive hematopoiesis of all lineages. In addition, *runx2*$^{(-/-)}$ mice die just after birth due to an inability to breathe because of a complete lack of ossification (Komori et al., 1997). This suggests that *RUNX2* plays an essential role in osteogenesis. *Runx3*$^{(-/-)}$ mice also die soon after birth because of starvation caused by hyperplasia of gastric epithelial cells, which indicates that *RUNX3* acts as a suppressor of gastric cancer (Li et al., 2002). Since *rnt-1* is the only homologue found in *C. elegans*, it is likely to be essential for *C. elegans* development. Supporting this notion, RNA interference targeted to *rnt-1* causes approximately 30% embryonic lethality and larval lethality (Nam et al., 2002). However, the *rnt-1(ok351)* mutant animals did not show any embryonic or larval lethality. We suggest that the in-frame deletion of 38 amino acids, including 9 amino acids of the Runt domain, which results in a mutant protein that lacks the C-terminal DNA-binding loop, does not completely knock out the function of RNT-1. It is widely believed that the sequence-specific DNA binding of

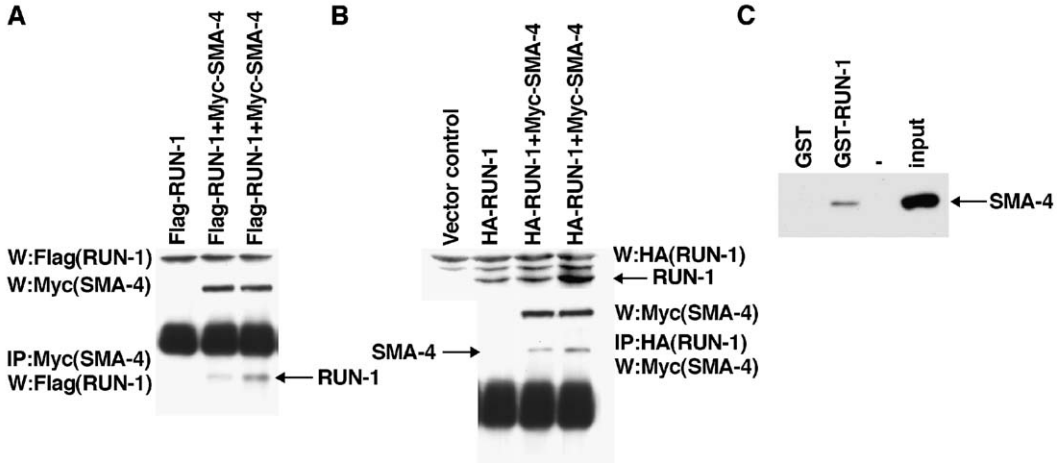

Fig. 5. Physical interaction of RNT-1 with SMA-4. (A–B) *sma-4* Tagged with the Myc epitope and *rnt-1* tagged with HA or Flag epitope were transiently transfected into 293 cells, and protein extracts obtained from the cells were analyzed by immunoprecipitation and Western blotting using the indicated antibodies. (C) GST–RNT or GST alone were incubated with a protein extract of 293 cells that had been transfected with a Myc-tagged SMA-4 expression plasmid. Ten percent of the protein extract used for the assay was applied as control (input). Myc-SMA-4 protein was detected by Western blotting using an anti-Myc antibody.

transcriptional factors to cognate *cis*-regulatory sequences on their downstream target genes is essential for their function during development. However, several lines of evidence from both *Drosophila* and mouse model systems suggest that the DNA-binding activity of a transcriptional factor is not essential for its in vivo function (Wheeler et al., 2002). An example of a DNA-binding defective mutant that retains significant in vivo activity is *Drosophila* Runt, as this protein represses the engrailed whether its DNA-binding activity is intact (Wheeler et al., 2002). Similarly, *rnt-1(ok351)* may produce an RNT-1 protein that is only partially inactivated and that retains the DNA binding-independent activity of the wild-type protein. It is also supported by the fact that *rnt-1(ok351)* is a little longer than the Sma/Mab pathway mutants.

Nevertheless, the mild but characteristic phenotypes of *rnt-1(ok351)* have made our study of the genetic interaction of *rnt-1* with other known components of the TGF-β signaling pathway possible, and we believe that it will be extremely useful in helping us to identify the other genetic components for regulating body length or male tail development in *C. elegans*.

Is rnt-1 involved in the Sma/Mab TGF-β signaling?

The TGF-β signaling pathways appear to be well conserved throughout evolution, and the genes involved in the TGF-β signaling pathways of *C. elegans* have been well characterized (Patterson and Padgett, 2000). *C. elegans* appears to have at least three genetic pathways that have been proven to deliver the TGF-β signal, namely, the *daf-7*, the *dbl-1*, and the *unc-129* pathways. The *dbl-1* pathway regulates body length and male tail development, and hence this pathway is also called the Sma/Mab pathway (Sma for the phenotype of small body size and Mab for the phenotype of male abnormal phenotype). Many components of this pathway have been identified, and these include the type I receptor SMA-6 (Krishna et al., 1999), the type II receptor DAF-4 (Estevez et al., 1993), the TGFβ-like ligand DBL-1 (Morita et al., 1999), and the cytosolic Smads SMA-2, -3, and -4 (Savage et al., 1996). These mutants have identical phenotypes which all show small body size and male tail defects.

The *rnt-1(ok351)* mutants show small body size and male tail defects, which are similar phenotypes of the Sma/Mab pathway mutants, and the spatial expression pattern of *rnt-1* gene also overlaps with that of the genes in the Sma/Mab pathway. Moreover, numerous reports indicate that mammalian homologues, RUNXs, interact with Smads in TGF-β signaling (Hanai et al., 1999; Alliston et al., 2001; Zhang et al., 2000). These facts urged us to investigate the relationship between RNT-1 and the Sma/Mab pathway in *C. elegans*. As shown here, double mutants of *rnt-1(ok351)* with the Sma/Mab pathway mutants show synergistic effects to regulate the body length and male tail development. In addition, we have shown that RNT-1 physically interacts with SMA-4, which is one of the Sma/Mab pathway regulators. Our data suggest two possibilities. One possible interpretation is that RNT-1 is involved in regulation of body length and male tail development which is new and parallel with the Sma/Mab pathway. Genetic synergism, rather than epistasis, and slight differences of male tail defects between *rnt-1(ok351)* and the Sma/Mab pathway mutants support this idea. However, many lines of evidence from mammalian studies, which suggest that RUNX is the transcription factor in TGF-β signaling, weaken this possibility. The other possibility is that *rnt-1* is a transcription factor in the Sma/Mab TGF-β signaling pathway. First, the physical interaction between RNT-1 and SMA-4, which is the first biochemical evidence shown in the Sma/Mab pathway, supports this possibility. Second, when we carefully reexamined the *rnt-1(ok351);lon-1(e185)* double mutants, it appeared that *lon-1(e185)* is epistatic to *rnt-1(ok351)*, suggesting that *lon-1* could be a downstream target of *rnt-1*. Indeed, we have identified possible RNT-1-binding sequences at the 5′ - upstream of the *lon-1* gene. However, in contrast to the previous mutants of the Sma/Mab pathway which have shown classic genetic epistasis, *rnt-1* mutants show synergistic effects with these mutants in the pathway. Therefore, it is quite possible that RNT-1 has nonoverlapping function in addition to its role in TGF-β signaling, and we cannot rule out the possibility that *rnt-1* and the Sma/Mab pathway act parallel to regulate the body size and male tail development. We hope that further characterization of the *rnt-1(ok351)* or other alleles of *rnt-1* mutants will resolve this argument.

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